Journal of Pest Science (2025) 98:2177–2189
https://doi.org/10.1007/s10340-025-01932-z

RNAi-mediated suppression of embryos as a promising strategy to control *Spodoptera littoralis*

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Received: 7 August 2024 / Revised: 23 May 2025 / Accepted: 27 May 2025 / Published online: 5 August 2025

Abstract
RNAi for insect control is a promising alternative to synthetic insecticides. Intense research efforts over the years have allowed researchers to develop effective control strategies and, recently, the registration of a new product for the US market. To date, however, the insect stages targeted by RNAi are both juveniles and adults, while the egg stage has been largely ignored, although an early suppression of the pest would more efficiently limit its damage. Here we try to fill this gap by focusing on the silencing of *Sl102*, a gene that encodes precursors of functional amyloid fibrils involved in the immune response and that, based on literature reports, could have an important role in the modulation of the embryonic development of lepidoptera. We showed that *Sl102* is expressed throughout the embryogenesis of *Spodoptera littoralis*, showing a peak 32 h after oviposition. The transcription level of this gene is strongly reduced by RNAi induced by soaking the eggs in a dsRNA solution. Interestingly, gene silencing is associated with a drastic reduction in egg hatching rate, which is complemented by a very high mortality of the few hatched larvae. Structural and ultrastructural analyses showed a significant delay in the development of silenced embryos, which also exhibited morphological alterations. Our results expand the understanding of the *Sl102* gene function, indicating an important role in embryonic development that remains to be studied from a functional point of view. This paves the way toward the future development of effective control strategies for *S. littoralis*, based on the suppression of embryonic development through RNAi technology.

Keywords Lepidoptera · Insect control · RNAi · Immunity · Embryonic development

Introduction

Lepidoptera are among the most harmful pests in agriculture, causing important damages to a wide range of crops. Considering their ability to develop resistance to different insecticides, including the *Bacillus thuringiensis* (*Bt*) toxins (Matten et al. 2008), and the increasing demand for alternative pest control tools imposed by EU policies aimed at achieving a drastic reduction of pesticide use in agriculture (Gohin 2024), it is imperative to develop new sustainable technologies for controlling these key-pests of many crops.

The use of RNAi for pest control was proposed more than 15 years ago (Mao et al. 2007), yet despite considerable research efforts, the first commercial product as a

Communicated by Nicolas Desneux.

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foliar spray only recently reached the market (Rodrigues et al. 2021). However, more spray-induced gene silencing (SIGS) products are expected to be marketed, since they allow overcoming the acceptance problems associated with the use of GMOs (Genetically Modified Organisms) for the delivery of dsRNAs.

However, RNAi efficiency in Lepidoptera is quite variable (Terenius et al. 2011; Joga et al. 2016), because dsRNAs can be quickly degraded by nucleases present in the saliva, gut juice, and hemolymph (Garbutt et al. 2013; Guan et al. 2018). To alleviate the dsRNAs degradation issue, different delivery and formulation strategies have been developed (Christiaens et al. 2018; De Schutter et al. 2021). However, there are also studies on Lepidoptera reporting effective RNAi induction by oral delivery of dsRNA (Turner et al. 2006; Mao et al. 2007; Surakasi et al. 2011; Wang et al. 2011; Di Lelio et al. 2014, 2022; Caccia et al. 2016, 2020). One strategy for enhancing the success of RNAi in Lepidoptera, and more generally in insects, is to target very early developmental stages, such as embryos and eggs, which present a less harsh degradation environment for dsRNAs and would allow an earlier pest suppression. However, limited information is available on suitable target genes to be silenced for pursuing this objective.

The study of the molecular interaction between pest insects and their Hymenoptera parasitoids is an important but poorly explored source of genes and molecules that may be used in insect control (Pennacchio et al. 2012). Indeed, virulence factors encoded by wasps or by associated symbiotic viruses in the family Polydnaviridae can disrupt vital functions, influencing, among others, the neuroendocrine balance and the immune barriers of their hosts (Pennacchio and Strand 2006). In our studies on the mechanisms underlying immune suppression in larvae of the tobacco budworm, *Heliothis virescens* (Lepidoptera, Noctuidae), by the braconid wasp *Toxoneuron nigriceps* (Hymenoptera, Braconidae), we discovered a host gene, named *102*, that encodes a protein (P102) producing functional amyloid fibers involved in the immune response and that is strongly downregulated in parasitized *H. virescens* larvae (Falabella et al. 2012). A homologous gene has also been found in the related noctuid moth species *Spodoptera littoralis* (*Sl102*) (Di Lelio et al. 2014) and *Trichoplusia ni* (Pascale et al. 2014). The latter study provided experimental evidence that P102 is part of a large group of endoribonuclease-U orthologs present in lepidopteran species, characterized by residual weak enzymatic activity and the capacity to produce amyloid fibers (Pascale et al. 2014). These amyloids, by coating non-self bodies, generate a structural scaffold promoting the encapsulation of invading organisms and a strictly localized polymerization of melanin on their surface (Falabella et al. 2012). Notably, the RNAi-mediated silencing of this gene generates an immunosuppressed phenotype in *S. littoralis* larvae, which makes them remarkably more susceptible to the septicaemia induced by *B. thuringiensis* and enhances the killing activity of this microbial biocontrol agent (Di Lelio et al. 2014; 2022; Caccia et al. 2016; 2020). The amyloid fibers produced by P102 are present in the cisternae of the rough endoplasmic reticulum of *H. virescens* hemocytes, where the *102* gene is highly transcribed (Falabella et al. 2012). These findings correlated with several studies reporting the occurrence of large vesicles in hemocytes, filled with notable amounts of fibrillar/tubular material, which, after secretion, accumulates in immune capsules and significantly contributes also to the formation of the basal lamina in epithelial tissues (Beaulaton 1968; Akai and Sato 1973; Wigglesworth 1973; Sass et al. 1994). Immunodetection studies demonstrating the presence of shared molecular epitopes in the basal lamina and hemocytes, further reinforced this ultrastructural evidence (Nardi and Miklasz 1989; Sass et al. 1994).

Based on this information, we hypothesized that the Sl102 protein, in addition to its role in the insect immune response, could also be involved in the formation of the basal lamina of the epidermis and other internal epithelia. Disruption of this function could induce alterations that negatively affect the fitness. To address this hypothesis and its relevance for successful embryonic development, we first assessed the temporal profile of *Sl102* gene expression in the embryo, then we developed a successful RNAi-based protocol to silence the *Sl102* gene during *S. littoralis* embryogenesis, and scored its impact on survival, development, and tissue differentiation. The lethal effect induced by gene silencing caused the disruption of embryo development and a precocious pest suppression that, by preventing the damage determined by feeding larvae, offers an effective new option for controlling this important pest insect.

Materials and methods

Insect rearing and egg collection

Spodoptera littoralis larvae were reared on an artificial diet (41.4 g/L wheat germ, 59.2 g/L brewer's yeast, 165 g/L corn meal, 5.9 g/L ascorbic acid, 1.53 g/L benzoic acid, 1.8 g/L methyl-4-hydroxybenzoate, and 29.6 g/L agar) at 25 ± 1 °C, 70 ± 5% RH, and photoperiod of 16:8 h light/dark until pupation. The emerging adults were reared in a cylindric glass jar (16 cm diameter, 27 cm height), and fed with water/honey solution (50:50), allowing them to mate for 24 h. The presence of egg masses was checked every 30 min, and highly synchronized newly laid eggs, from the same egg mass, singly separated with a brush, were selected to define an experimental group for the following experiments.

Effect of gene silencing on embryo survival

Gene silencing was performed by soaking eggs collected from the same egg mass in PBS (Phosphate Buffered Saline 1×; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) containing 250 ng/µL dsRNA directed against the *Sl102* gene (ds*Sl102*) or, as control, against *GFP* sequences (ds*GFP)*, produced as described in Di Lelio et al. (2022). This procedure of dsRNA delivery through the egg barriers to the developing embryo was used given its efficacy in the induction of RNAi observed for another lepidopteran species, *Ostrinia furnacalis* (Wang et al. 2011), as well as in Coleoptera (Pampolini et al., 2020), Diptera (Al-Behadili et al. 2024) and mites (Yang et al. 2024; Wang et al. 2025). Briefly, 120 highly synchronized eggs, laid within a 30-min time interval, were collected as described above in a 1.5 mL Eppendorf tube and soaked in 50 µL of *dsSl102* or ds*GFP* solution at 25 °C ± 1. To assess the impact of the soaking duration and of any dose-dependent effect of dsRNA treatment on embryo survival, we carried out soaking experiments of three different durations (30, 60, and 120 min) and for each of them we used three different concentrations of dsRNA (50, 100 and 250 ng/µL). Embryo survival was determined by recording the number of hatched eggs. In all subsequent experiments of gene silencing, the soaking was carried out using the combination of duration-concentration which had the highest impact on embryo survival (120 min, 250 ng/µL).

Total RNA extraction

Total RNA was extracted from *S. littoralis* eggs using TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions and stored at −80 °C. The concentration and purity of total RNA were determined using a Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). For absolute quantitative Real-Time PCR (qRT-PCR) analyses, RNA samples were diluted to a concentration of 200 ng/µL.

Production of a standard curve for absolute quantification by qRT-PCR

Total RNA extracted from *S. littoralis* eggs was subjected to retrotranscription with the Ambion® RETROscript® Kit (Thermo Fisher Scientific) and then used for PCR amplification of a 111 bp *Sl102* fragment, with specific primers (Table 1). The PCR products were cloned in the pCR4-TOPO TA Vector (Thermo Fisher Scientific), which was introduced into chemically competent One Shot® TOP10 *Escherichia coli* cells (Thermo Fisher Scientific) and plated on LB agar. Plasmids from colonies grown overnight were extracted using GRS Plasmid Purification Kit – Mini (Grisp Research Solutions, Porto, Portugal) and sequenced. The standard curve was prepared by plotting the logarithm of eight tenfold dilutions of a starting solution containing 10 ng/µL (number of copies: about 3×10^9) of the obtained plasmid against the corresponding Ct values. The PCR efficiency was calculated based on the slope and the coefficient of correlation (R^2) of the standard curve, according to the following formula: $E = 10^{(-1/\text{slope})} - 1$ ($E = 109.41$; slope = −3.115; intercept = 13.351; $R^2 = 0.9987$).

Table 1 Primer pairs used for qRT-PCR analysis

Oligos ID	Sequence 5' – 3'	Amplicon size (bp)	Tm (°C)
RT*SlActin*Fw	CCGTCTTCCCATCCATCGT	66	60
RT*SlActin*Rv	CCTTCTGACCCATACCAACCA		
RT*Sl102*Fw	GGCGGTGTCGTCGATTAT	111	60
RT*Sl102*Rv	ATTGAACATTTCCTCGCTC		
q*GFP*2f	CGCCACAACATTGAAGATGGA	88	60
q*GFP*2r	CTGGTAAAAGGACAGGGCCA		

Sl102 expression by qRT-PCR analysis

The relative expression of genes was measured by one-step qRT-PCR, using the Power SYBR™ Green RNA-to-CT™ 1-Step Kit (Applied Biosystems), according to the manufacturer's instructions, with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001; Pfaffl 2001), using *Sl102* gene-specific primers and *SlActin* gene-specific primers as internal reference (Tab. 1), as described in Di Lelio et al. (2014).

For the validation of the $2^{-\Delta\Delta CT}$ method the difference between the Ct value of *Sl102* and the Ct value of *SlActin* transcripts [ΔCt = Ct(*Sl102*) − Ct(*SlActin*)] was plotted versus the log of tenfold serial dilutions (5000, 500, 50, 5 and 0.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope less than 0.1 (Slope = 0.0154, $R^2 = 0.0776$), indicating that the efficiencies of the two amplicons were approximately equal.

The internal reference resulted stable across the conditions used at all in different experimental sets (Supplementary Fig. S1 and Supplementary Table S1).

Time-course analysis of *Sl102* gene expression

Spodoptera littoralis eggs were collected at nine time points: 8, 16, 24, 32, 40, 48, 56, 64, and 72 h after eggs laying. For each time point, 6 pools of 20 eggs were collected and processed for total RNA extraction as described above. The obtained RNA samples were used for qRT-PCR analyses,

using the Applied Biosystems™ Power SYBR™ Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The quantity of transcripts in the samples was determined by relating the obtained Ct values to the established standard calibration curve, according to the absolute quantification method (Rutledge and Côté, 2003).

Sl102 gene silencing by egg soaking

Sl102 gene silencing was performed by soaking eggs in an 1.5 mL Eppendorf tube as described above. Briefly, 120 highly synchronized eggs, were soaked in 50 µL of ds*Sl102* or ds*GFP* solution (250 ng/µL) for 15, 30, 60, and 120 minutes at 25°C ± 1, to assess the impact of treatment duration on gene silencing efficiency. Eggs were washed twice with 100 µL of 1× PBS for one minute, transferred to a Petri dish filled with a sterile filter paper disk, separated with a paint brush, and checked for integrity under a stereomicroscope. Damaged eggs were discarded. After 48 hours, for each experimental time point, 20 eggs were collected in a 1.5 mL Eppendorf tube and processed as described above to obtain the total RNA for the relative expression analysis. The experiments were replicated 10 times.

Finally, the expression of *Sl102* in *S. littoralis* eggs was quantified at different time points after soaking in ds*Sl102 or* ds*GFP* solutions for 120 min, as described above. From each experimental condition 6 groups of 20 eggs were collected in 1.5 mL Eppendorf tubes and stored at the rearing temperature until RNA extraction, which was carried out 8, 16, 26, 34, 50, and 74 h after soaking, as described above. Total RNA was used for relative expression analysis (see below). The experiment was replicated 9 times.

Assessment of dsRNA internalization in *S. littoralis* eggs

To demonstrate the entry of dsRNA into *S. littoralis* eggs after soaking treatment, two different experiments were set up: a) eggs were soaked for 120 min in a ds*GFP* solution and the delivery was monitored by qRT-PCR; b) eggs were soaked for different time intervals (15, 30, 60, and 120 min) in a solution containing fluorescently labeled ds*Sl102*, obtained by using the Silencer™ siRNA Labeling Kit with Cy™3 dye (Thermo Fisher Scientific) according to the manufacturer's instructions, and the possible internalization was monitored by fluorescence quantity analysis. Newly laid eggs were collected within a 30-min time interval and transferred to a 1.5 mL vial containing 20 µL of ds*GFP* (about 200 ng/µL) or ds*Sl102*-labeled (250 ng/µL) solutions. Following exposure, the dsRNA solution was withdrawn, and the eggs were washed four times with PBS to remove dsRNA present on the surface. For experiment "a", wash supernatants were separately collected and stored at –80 °C for subsequent RNA extraction. Post-washing, the eggs were then randomly split into two equal groups (20 eggs per group). One batch of eggs was subjected to chorion removal by adding 3% sodium hypochlorite for one minute, followed by three rinses with nuclease-free water, whereas the second group remained untreated. All samples were stored at −80 °C until RNA extraction. Total RNA was isolated from both washed eggs (dechorionated and non-dechorionated) and from wash fractions, using the TRIzol™-based extraction protocol described above. qRT-PCR analysis was performed as described above, using specific primers targeting an internal 88 bp region of the ds*GFP* molecules (Table 1), and the resulting Ct values were interpolated onto a previously established standard curve (slope = −2371; intercept = 17.266; $R^2 = 0.9845$) constructed by using known ds*GFP* concentrations, to quantify the internalized dsRNA. The presence of ds*GFP* within washed eggs, in comparison to the wash fractions, served as an indicator of successful dsRNA internalization. The experiment was conducted with five biological replicates. For experiment "b", eggs soaked for different time intervals in a solution of fluorescently labeled ds*Sl102* were visualized under a fluorescent stereomicroscope (Leica M205 FCA) before and after the washing step, and the fluorescence intensity was measured by using the ImageJ software (v1.54p 17 February 2025). Untreated eggs were used as a control (no expected fluorescence signals). For each experimental group, eight biological replicates were analyzed.

Developmental analysis of *S. littoralis* embryos treated with dsRNAs

The phenotypic alterations induced by *Sl102* gene silencing were characterized on *S. littoralis* highly synchronized eggs. *S. littoralis* eggs(140 - 280) were collected from the same egg mass and soaked in 50 µL of 250 ng/µL ds*Sl102* or ds*GFP* solution for each experimental condiction. After 120 min the eggs were washed, transferred to a Petri dish, and checked for integrity. From each experimental group, after 48 h, 20 eggs were transferred in a 1.5 mL Eppendorf tube and processed for RNA extraction. The total RNA was used for the relative expression analysis. The remaining eggs were allowed to develop in Petri dishes at the rearing conditions described above. Eggs were observed daily until hatching. For each experimental group, newborn larvae were placed in 4-well plastic rearing trays (RT32W, Frontier Agricultural Sciences, Pitman, NJ, United States), containing the artificial diet and closed with perforated plastic lids (RTCV4, Frontier Agricultural

Sciences), and daily checked for survival. The experiment was replicated 5 times.

Light and transmission electron microscopy (TEM)

72 h after dsRNA treatment, eggs were dissected in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The obtained embryos were fixed with the same solution for 2 h, washed three times for 5 min each in 0.1 M sodium cacodylate buffer, and then postfixed with 1% osmium tetroxide (diluted in the same buffer) for 2 h at room temperature. After dehydration in ascending ethanol series, embryos were embedded in Epon-Araldite 812 mixture resin (Sigma-Aldrich, Milan, Italy). Sections were obtained with a Reichert Ultracut S ultramicrotome (Leica, Wien, Austria). Semi-thin Sects. (0.6-µm-thickness) were stained with crystal violet and basic fuchsin and observed with a Nikon Eclipse Ni microscope (Nikon, Tokyo, Japan) equipped with a DS-5 M-L1 digital camera system (Nikon). Thin Sects. (70-nm-thickness) were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a JEOL-1010 transmission electron microscope (TEM) (Jeol, Tokyo, Japan) equipped with a Morada digital camera (Olympus, Tokyo, Japan)—CRIETT, University of Insubria. Densitometric analysis of the basal lamina was performed with Fiji (ImageJ). After converting TEM images (randomly taken at × 80,000 magnification) to 8-bit, three regions of interest (ROI) were selected within the basal lamina of the midgut epithelium (5 images per sample were analyzed, three ROIs were selected in each image). The "threshold" was set with the thresholding tool and total pixels were measured. Results were expressed as arbitrary densitometry units (ADU).

Statistical analysis

The effect of dsRNA treatment on egg hatching in relation to soaking duration was analyzed using a two-way ANOVA, considering dsRNA treatment and incubation time as independent factors. *Sl102* gene expression after gene silencing in egg soaking experiments was analyzed by two-way ANOVA, considering the effects of dsRNA treatment and time post-treatment. The cuticle thickness and basal lamina density of embryos were analyzed by unpaired Student's *t*-test. The survival rate of embryos and newborn larvae was analyzed by using the Log-rank (Mantel-Cox) test. ds*GFP* quantity after soaking treatment was analyzed by Kruskal–Wallis test. When significant effects were observed (P-value < 0.05), Dunn's multiple comparisons test was used. Fluorescence intensity analysis after soaking treatment was analyzed by one-way ANOVA test. The normality of all the data was checked using the D'Agostino & Pearson's test, while Brown-Forsythe's test was carried out to test the homogeneity of variance. When significant effects were observed (P-value < 0.05), Tukey's test was used for multiple comparisons test. All the data were analyzed using GraphPad Prism (version 6.0).

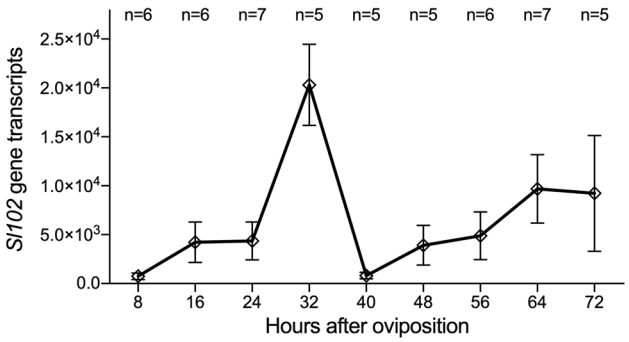


Fig. 1 Levels of *Sl102* gene transcripts during embryogenesis expressed as number of RNA molecules. The values reported are the mean ± standard error of the mean (SEM). The sample size is denoted by *n*. See supplementary table S2 for punctual values reported in the graph

Results

Expression profile of the *Sl102* gene during embryonic development and its silencing by RNAi

The *Sl102* gene is expressed during the embryonic development of *S. littoralis* (Fig. 1), showing a sharp peak at 32 h after oviposition, followed by a sudden decrease and then a gradual increase over time between 40 and 64–72 h.

We then assessed the impact of gene silencing on embryo survival, using different combinations of soaking duration and dsRNA concentrations. There was a clear dose-dependent response, increasing over time, with the highest negative impact on embryo survival observed when soaking was carried out for 120 min, using a dsRNA concentration of 250 ng/µL (Fig. 2). The results demonstrated that soaking treatment with ds*Sl102* led to a significant reduction in egg hatching compared to controls (Two-way ANOVA: P-value < 0.0001). This effect was influenced by both dsRNA concentration (Two-way ANOVA: P-value < 0.0001) and soaking duration (Two-way ANOVA: P-value < 0.0001). Based on these findings, for subsequent experiments aimed at evaluating gene expression, a dsRNA concentration of 250 ng/µL and a soaking time of 120 min were selected, as they resulted in the strongest reduction in egg hatching.

To demonstrate that the observed strong reduction of survival rate was associated with a significant level of gene silencing, we measured the *Sl102* expression profile (qRT-PCR) 48 h after soaking treatments of increasing duration

(15, 30, 60, and 120 min) and, at concentration of dsRNA of 250 ng/µL. The level of gene silencing increased as a function of the soaking time, reaching its maximum when the eggs were treated with the dsRNA solution for 2 h (Two-way ANOVA: $P < 0.0001$, Fig. 3).

This soaking time induced significant gene silencing, which started 14 h after the dsRNA treatment and persisted throughout a 72-h time-course experiment (Two-way ANOVA: P-value < 0.0001) (Fig. 4).

Evidence of dsRNA entrance in *S. littoralis* eggs

To further corroborate the functional link between gene silencing induced by RNAi, we carried out additional experiments to demonstrate the penetration of dsRNA in *S. littoralis* eggs. qRT-PCR analysis confirmed the presence of ds*GFP* in both experimental groups (eggs before and after chorion removal), with significantly higher concentrations detected in egg groups compared to the fourth wash fraction ($P < 0.05$) (Fig. 5a). The absence of significant differences between the dechorionated and non-dechorionated egg groups further validated the effective internalization of dsRNA. The ds*GFP* concentration decreased in wash steps 1–4, indicating that the washing protocol gradually removed surface-bound dsRNA, although the differences were not statistically significant.

Furthermore, as demonstrated in Fig. 5b, for each time interval of the egg-soaking treatment, unwashed eggs showed similar fluorescence intensity signal, indicating that eggs were uniformly overlaid by labeled-dsRNA molecules and no differences could be detected by the analysis. Nevertheless, after washing eggs, a gradual increase in fluorescence intensity was observed, and it was proportional to the increase in soaking time. The differences between the different groups analyzed are statistically significant (P-value < 0.05). Notably, fluorescence intensity of 120-min-soaked eggs, after washing step, was comparable to that of unwashed eggs, suggesting that this time interval is the most suitable for the delivery of dsRNA molecules, as previously demonstrated by gene silencing analysis (see Fig. 2).

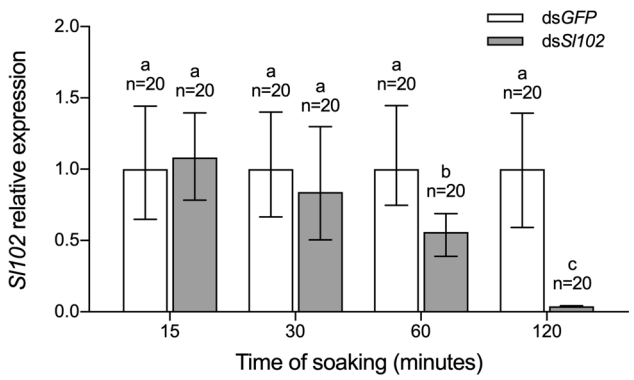


Fig. 3 Relative expression of the *Sl102* gene in *Spodoptera littoralis* eggs, 48 h after soaking in a dsRNA solution (250 ng/µL) for different time intervals. Different letters denote a significant difference between mean values recorded for each soaking time (Two-way ANOVA: $F_{(5,108)} = 134.5$, P-value < 0.0001, $n = 10$). Both the soaking treatment with ds*Sl102* (Two-way ANOVA: $F_{(1,152)} = 138.4$, P-value < 0.0001) and the time of treatment (Two-way ANOVA: $F_{(3,152)} = 66.49$, P-value < 0.0001) significantly affected the level of the *Sl102* transcripts. The values reported on the y-axis indicate relative expression ± SEM. The sample size is denoted by n and different letters denote significant differences in the Tukey's multiple comparisons test (P-value < 0.0001). See supplementary table S3 for punctual values reported in the graph

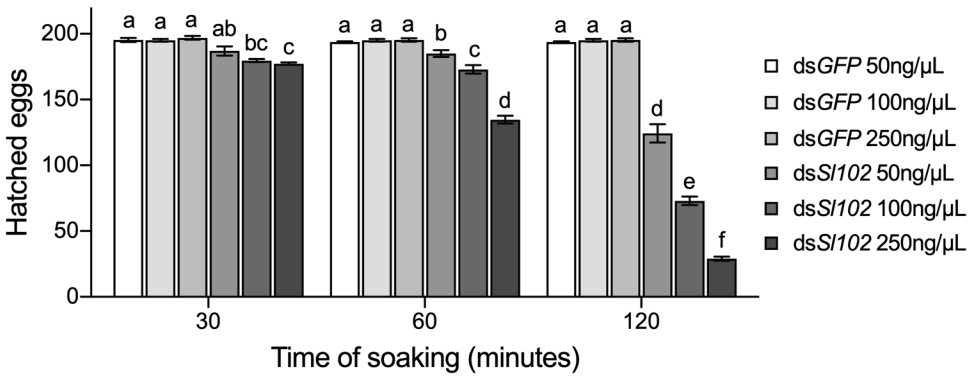


Fig. 2 Hatching rate of *Spodoptera littoralis* eggs following soaking treatment of different duration (30, 60, and 120 min) with varying concentrations of ds*Sl102*. The significant reduction in egg hatching compared to controls observed following ds*Sl102* soaking treatment (Two-way ANOVA: $F_{(10,36)} = 183.4$; P-value < 0.0001, $n = 600$) was influenced by both dsRNA concentration (Two-way ANOVA: $F_{(2,36)} = 783.0$; P-value < 0.0001) and soaking duration (Two-way ANOVA: $F_{(5,36)} = 557.6$; P-value < 0.0001). Data are presented as mean ± SEM. Different letters indicate statistically significant differences according to Two-way ANOVA (P-value < 0.0001)

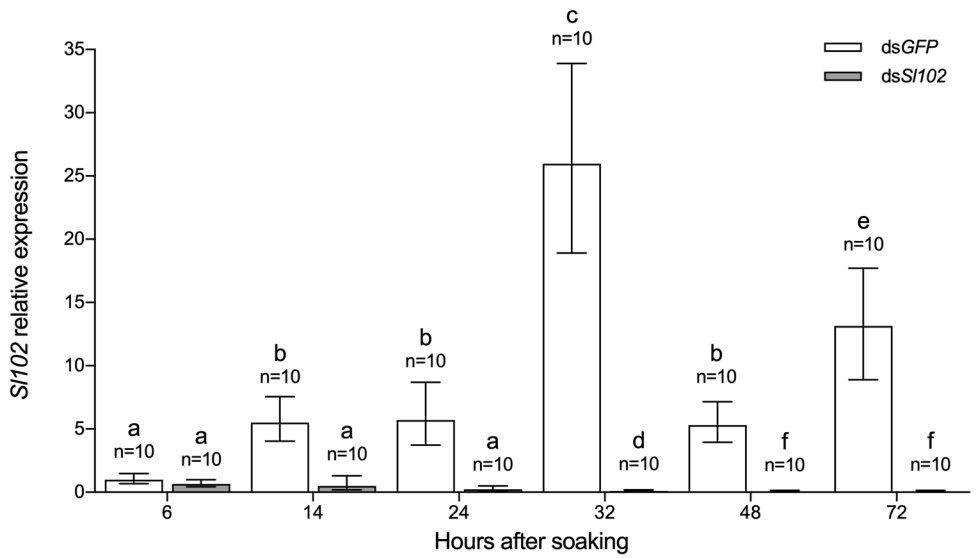


Fig. 4 *Sl102* gene silencing in *Spodoptera littoralis* eggs at different times after dsRNA treatment. The level of *Sl102* gene transcripts resulted significantly reduced starting from 14 h post-treatment (Two-way ANOVA: $F_{(5,108)} = 134.5$, P-value < 0.0001, n = 10). Both the soaking treatment with ds*Sl102* (Two-way ANOVA: $F_{(1,108)} = 2076$, P-value < 0.0001) and the time post-treatment (Two-way ANOVA: $F_{(5,108)} = 54.23$, P-value < 0.0001) significantly affected the level of the *Sl102* transcripts. The values reported are the mean ± standard error of the mean (SEM). The sample size is denoted by *n*. Different letters denote significant differences in Tukey's multiple comparisons test (P-value < 0.0001). See supplementary table S4 for punctual values reported in the graph

Analysis of phenotype changes induced by *Sl102* gene silencing

The significant reduction of *Sl102* gene expression documented in *S. littoralis* eggs soaked in ds*Sl102* solution (Fig. 6a) was associated with a dramatic decrease of the hatching rate (Log-rank test: P-value < 0.0001). Only 5% of ds*Sl102*-treated eggs hatched, compared to 84% hatching rate recorded for control eggs (Fig. 6b). Moreover, the few larvae that successfully hatched from ds*Sl102*-treated eggs showed a significantly lower survival rate than controls (Log-rank test: P-value < 0.0001) (Fig. 6c). Developmental abnormalities became apparent at 72 h post-oviposition in *Sl102*-silenced embryos, including reduced sclerotization of the head capsule and delayed formation of the larval gut; these alterations became even more evident at 96 h, when tissue degeneration was clearly apparent (Fig. 6d).

To better define the observed phenotypic alterations, we carried TEM observations. The effect of *Sl102* silencing on *S. littoralis* embryos was evaluated through morphological analysis performed 72 h after oviposition. A general developmental delay in ds*Sl102* embryos (Fig. 7b) compared to controls (ds*GFP*) (Fig. 7a) was clearly visible. Specifically, delayed differentiation of midgut cells was evident in ds*Sl102* embryos, along with the presence of yolk granules in the midgut lumen (Fig. 7b). The ultrastructural analysis of the midgut epithelium corroborated the hypothesis of a delay in the embryonic development in silenced embryos. In fact, although a large basal nucleus was present in goblet cells of both samples, the goblet cavity in ds*Sl102* embryos was just beginning to form (Fig. 7d), compared to ds*GFP* embryos, where it appeared completely differentiated and covered by microvilli (Fig. 7c). In addition, the apical membrane of columnar cells showed a well-developed brush border in controls (Fig. 7e), while microvilli were absent in ds*Sl102* embryos (Fig. 7f). Also the basal lamina exhibited a different morphology in the two embryos, with a less dense structure in ds*Sl102* individuals compared to controls (Fig. 7g-i). Finally, although an electron-dense epicuticle was produced in both experimental conditions (Fig. 7j, k), *Sl102* gene silencing affected the procuticle development and its thickness, which was significantly lower in silenced embryos compared to controls (Fig. 7j-l).

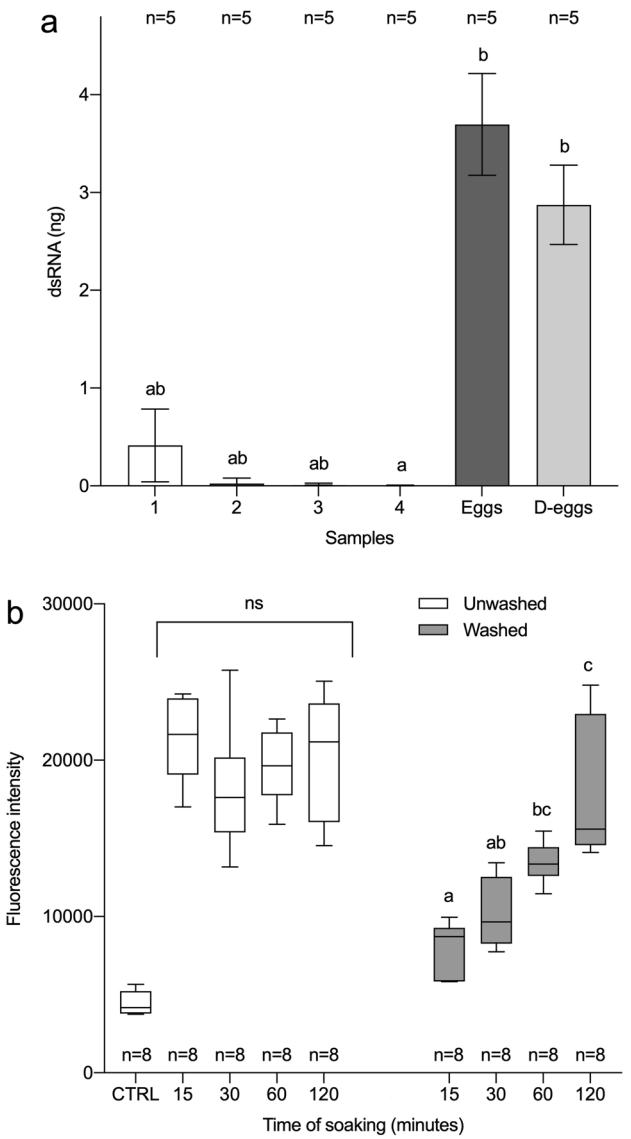


Fig. 5 **a** Quantification of ds*GFP* in *S. littoralis* eggs and wash fractions. Amounts of ds*GFP* (ng) detected in sequential wash fractions (1–4), non-dechorionated eggs (Eggs), and dechorionated eggs (D-Eggs) after a 2-h soaking in ds*GFP* solution. dsRNA levels were significantly higher in both egg groups compared to the final wash fraction (Kruskal–Wallis; K-W statistic = 24.12; *P*-value = 0.0002), confirming internal uptake. **b** Fluorescence intensity analysis of *S. littoralis* eggs soaked in labeled-ds*Sl102* solution for different time intervals. CTRL is the control group (untreated eggs); unwashed eggs are represented by the white rectangles. No significant differences between unwashed egg groups were observed (One-way ANOVA: *P*-value > 0.05); washed eggs are represented by the gray rectangles. Significant differences between washed egg groups were observed (One-way ANOVA: $F_{(8,63)} = 2.127$, *P*-value < 0.0001). The values reported are the mean ± SEM. The sample size is denoted by n. Different letters denote significant differences in the multiple comparisons test adopted

Discussion

The reduction of synthetic pesticide use in agriculture is a key-target of the green transition toward a more sustainable future. The implementation of multiple approaches in Integrated Pest Management (IPM) plans is the core strategy, which, however, is still largely based on the use of synthetic pesticides (Mansfield et al. 2024). Alternative pest control tools are, therefore, highly needed to reduce the negative impact of agriculture on environment and health. Among the many options currently being used, RNAi-based pest control technologies are very promising, given their efficacy and the high level of specificity (Germing et al. 2025), largely limiting the unintended effects on non-target species, which can negatively affect the ecosystem services they provide. The efficacy of this pest control method is highly dependent on the delivery strategy using different nanoformulations aiming to protect the dsRNA from degradation (Quilez-Molina et al., 2024), but also on the availability of suitable target genes that induce lethal phenotypes when silenced, possibly in early developmental stages to prevent any damage that feeding stages cause. The present work has allowed the identification of a target gene as a suitable candidate for developing RNAi-based strategies to suppress the embryos of the lepidopteran species *S. littoralis*, a widespread plant pest.

The *102* gene, isolated for the first time in *H. virescens*, is highly expressed in the hemocytes of lepidopteran larvae and is involved in the modulation of immune response (Falabella et al. 2012; Di Lelio et al. 2014; Pascale et al. 2014). The experimental data presented here clearly support that its homologous in *S. littoralis* (*Sl102*) has also an important role in embryonic development. Indeed, the silencing of this gene in *S. littoralis* embryos generates a lethal developmental alteration. However, this negative effect does not result in evident cellular damage, but in a remarkable delay in cellular differentiation, which determines mortality of the embryos and of the very few larvae that will eventually hatch.

It is apparently not easy to interpret the dual role of this gene, but the molecular mechanism mediated by the encoded protein may account for such a functional diversity. The production of amyloid fibrils by P102 and/or its fragments, as well as by orthologs occurring in other lepidopteran species, has a central role in the immune response (Falabella et al. 2012; Di Lelio et al. 2014; Pascale et al. 2014). Indeed, these functional amyloid fibrils provide a molecular scaffold promoting haemocyte aggregation (i.e., encapsulation) and localized melanin synthesis on the surface of coated non-self bodies, preventing the diffusion of toxic precursors, which would be lethal for the insect mounting an immune response (Falabella et al. 2012). It is very likely that this

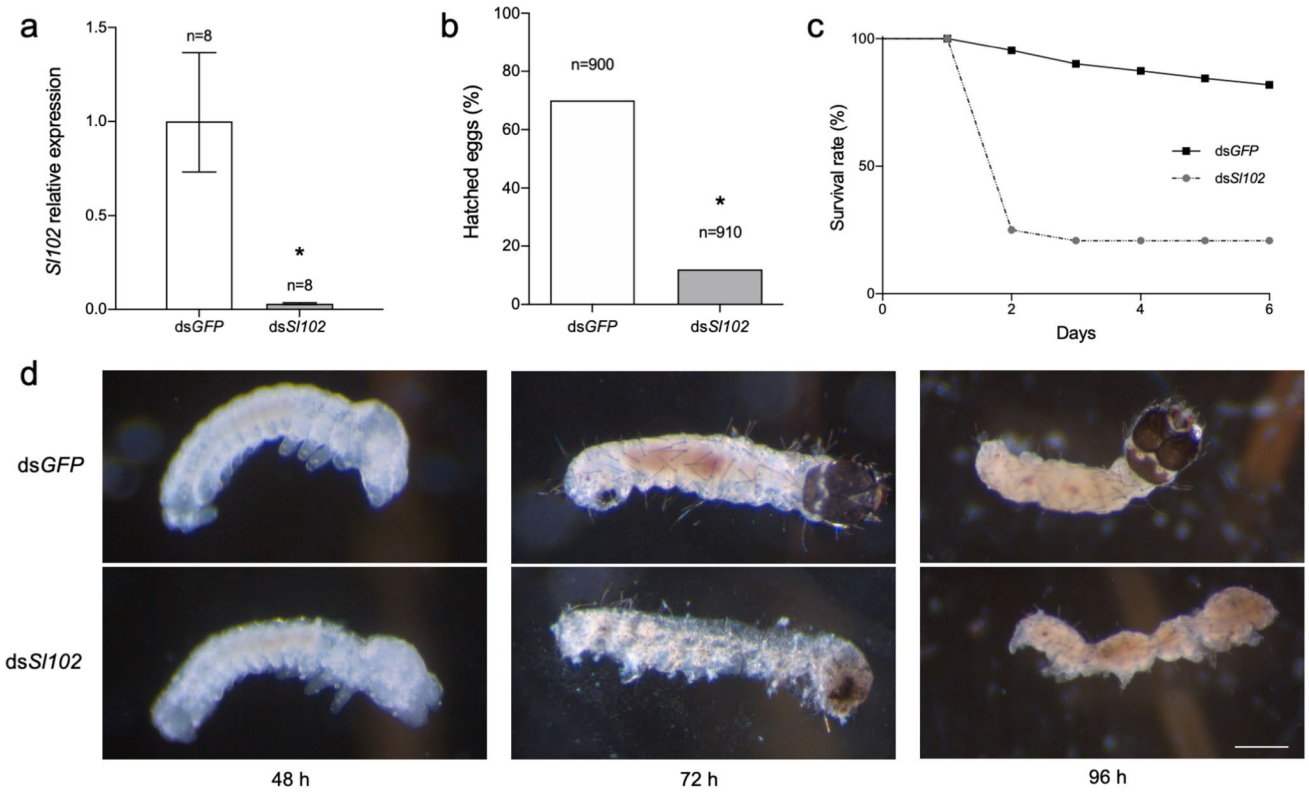
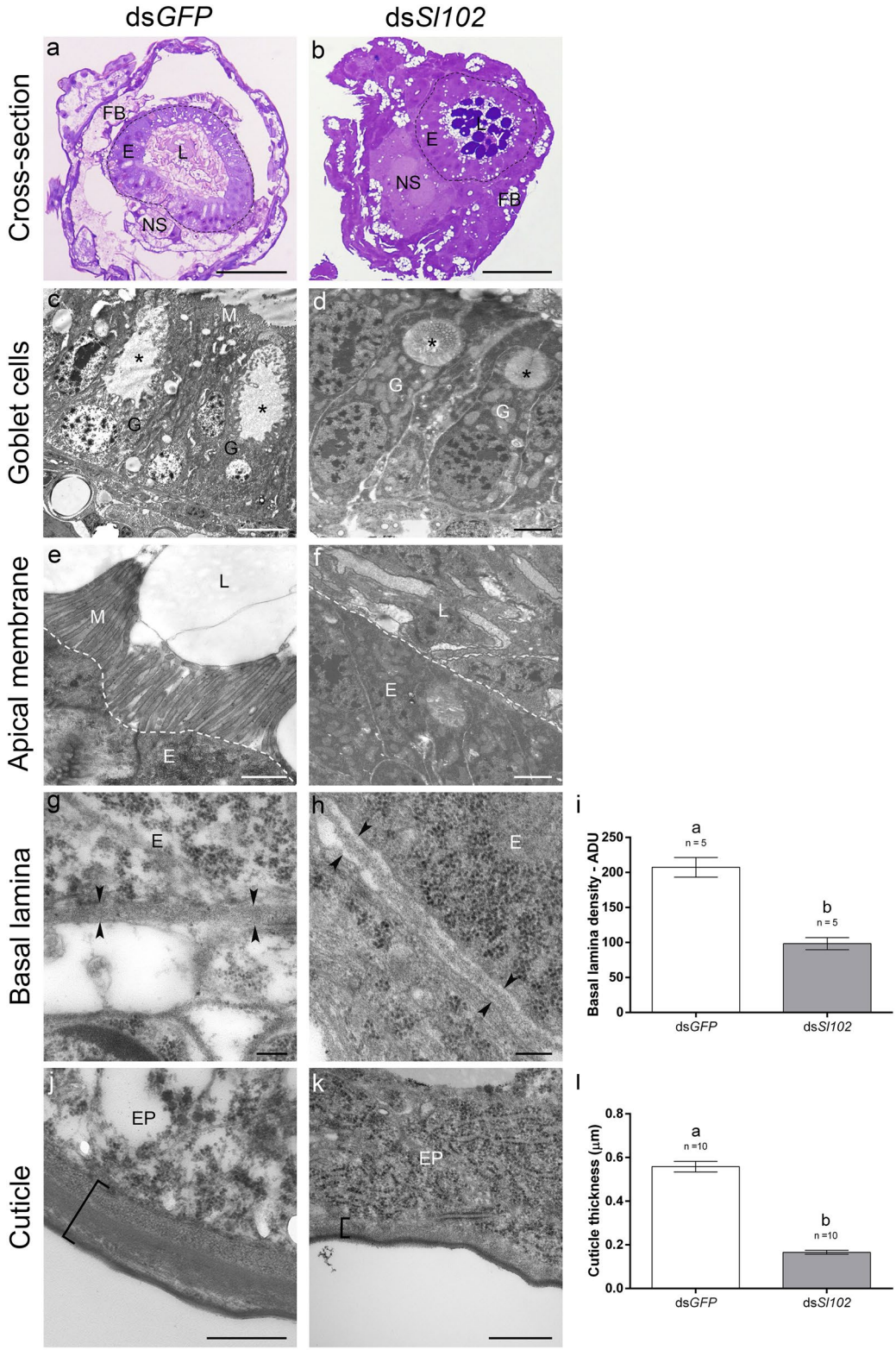


Fig. 6 Effects of *Sl102* gene silencing on egg and larval viability in *Spodoptera littoralis*. **a** Relative expression of the *Sl102* gene in eggs soaked in dsRNA solutions, presented as the mean ± standard error (SEM) (Student's t test: t = 24.97, df = 14, *P*-value < 0.0001). **b** Percentage of hatched eggs (embryo survival) in *GFP* (70%) and *Sl102* (12%) dsRNA-treated groups (Log-rank test: $\chi^2 = 209.9$; df = 1; *P*-value < 0.0001); **c** Survival rate of newborn larvae after the egg soaking treatment (Log-rank test: $\chi^2 = 133$; df = 1; *P*-value < 0.0001). **d** Stereomicroscope analysis of developing embryos at different time points following treatment with *dsSl102* and *dsGFP* (control) (scale bar = 200 µm). The sample size is denoted by n. See supplementary table S5 and S6 for detailed values reported in the graphs

master role played by P102 in orchestrating the immune response is operated not only against foreign invaders but also at wounding sites, where the disrupted basal membrane could mediate a localized clotting and melanisation reaction. This experimental evidence fits well with previous reports showing that hemocytes are involved in the formation of the basal lamina, where they release consistent amounts of secreted fibrillar material, found also in the immune capsules (Beaulaton 1968; Akai and Sato 1973; Wigglesworth 1973; Nardi and Miklasz 1989; Sass et al. 1994). It is interesting to note that this fibrillar material located in the cisternae of the endoplasmic reticulum of *H. virescens* hemocytes binds to P102 polyclonal antiserum, as demonstrated by immunogold labeling experiments (Falabella et al. 2012). Therefore, these fibrils are released and accumulate where functional amyloids regulate the complex array of localized immune reactions, which generate toxic defense compounds.

The role of hemocytes could be even more complex than producing these functional amyloid fibrils as constituents of the basal membrane of epithelia, since they could be also involved in the breakdown of the basal lamina, as observed in the developing wings of *Manduca sexta* (Nardi and Miklasz 1989). As the dynamic process of breakdown and formation of the basal lamina is necessary to allow the growth process, it is not surprising that the silencing of *Sl102* gene has such a strong impact on embryonic development, where fast cellular growth, division, and differentiation take place. This fascinating hypothesis received indirect support from the TEM observations showing the basal lamina of gut cells significantly less dense as a consequence of *Sl102* gene silencing. More in general, the observed developmental delay of embryos, which eventually largely fail to hatch, is associated with clear signs of delayed differentiation of gut cells and their impaired functionality. Yolk granules, which provide nutritional support to the developing


dsGFP
dsSl102
Cross-section
Goblet cells
Apical membrane
Basal lamina
Cuticle
a
b
c
d
e
f
g
h
i
j
k
l
FB
E
L
NS
M
G
EP
Basal lamina density - ADU
250
200
150
100
50
0
n = 5
n = 5
dsGFP
dsSl102
Cuticle thickness (µm)
0.8
0.6
0.4
0.2
0.0
n =10
n =10

◂**Fig. 7** Morphological comparison of ds*GFP* and ds*Sl102* embryos. **a**, **b** Cross-sections of the embryo; **c–h**, **j–k** TEM analysis; **i** Comparison of the basal lamina density; **l** Comparison of the cuticle thickness. The values reported are the mean ± SEM: **i** ds*GFP* = 207.299 ± 14.029; ds*Sl102* = 98.375 ± 8.629; **l**: ds*GFP* = 0.558 µm ± 0.024; ds*Sl102* = 0.166 µm ± 0.009. The obtained values passed normality test, Student's *t*-test: **i**: $t = 6.61$, df = 8, $P < 0.001$, n = 5; **l**: $t = 15.06$, df = 18, P-value < 0.0001, n = 10. Different letters denote significant differences (P-value < 0.0001). Arrowheads: basal lamina; asterisk: goblet cavity; black dashed line: midgut; bracket: cuticle; E: gut epithelium; EP: epidermis; FB: fat body; G: goblet cell; L: lumen; M: microvilli; NS: nervous system; white dashed line: apical membrane; Y: yolk granule. Bars: 50 µm (a, b), 5 µm (c), 2 µm (d, f), 1 µm (e), 500 nm (j, k), 200 nm (g, h)

embryo (Correia et al. 2013), were consistently present in the midgut lumen of silenced embryos at 72 h after oviposition, while, in contrast, they were absent in control embryos, where they were regularly processed and absorbed. These findings are further corroborated by TEM analysis, which showed the presence of goblet and columnar cells with a clear delay in differentiation. In particular, goblet cells of the silenced group presented a developing cavity above the nucleus, whereas a well-developed apical cavity was present in the controls. This is a clear signal of differentiation, as evidenced in *Manduca* embryos (Hakim et al. 1988). Moreover, the lack of microvilli in columnar cells of the gut in silenced embryos further support the developmental delay, since their presence signals differentiation in the midgut epithelial cells (van der Starre-van der Molen and de Priester, 1972). The cuticle thickness and stratification were negatively affected by *Sl102* gene silencing. This is due to the different development of the procuticle, which in the control group is structurally similar to that described in *Drosophila* and other insects (Moussian 2010). We can reasonably hypothesize that this is a consequence of the general developmental delay determined by *Sl102* silencing, which limits the differentiation of the cuticle, a process that starts around mid-embryogenesis, regardless of the length of the entire development (Moussian 2010). Our observations pave the way toward in-depth functional studies aiming to shed light on the molecular mechanisms underlying the role of *Sl102* in embryonic development, which is clear but remains to be studied. These functional studies will shed light on the mechanisms underlying the role of this gene in the embryonic development of *S. littoralis* and will allow to assess their possible generality in lepidoptera, given that homologue sequences are widespread in this insect order (Falabella et al. 2012; Di Lelio et al. 2014; Pascale et al. 2014).

The important role of *Sl102* in the modulation of immunity and development makes it an ideal candidate for developing RNAi-based pest control strategies, targeting both larvae and embryos. We have already indicated the use of *Sl102* silencing to generate an immunosuppressed phenotype that enhances the killing activity of *B. thuringiensis* (Di Lelio et al. 2014, 2022; Caccia et al. 2016; 2020). This allows to efficiently target larval stages, also increasing the susceptibility of later instars that are poorly sensitive to *Bt* toxins (Caccia et al. 2016). Here we further expand the exploitation of *Sl102* silencing for pest control by targeting the embryo stage. The observed high embryonic mortality looks very promising in terms of control efficacy, since it will drastically reduce the feeding damage of later stages and could nicely complement the abovementioned indirect killing effect on larval stages. However, to achieve this goal it is crucial to define adequate delivery strategies to maximize the impact on both larvae and embryos. This is a very active research area (Liu et al. 2020; Quilez-Molina et al., 2024). Of special interest for targeting lepidopteran embryos is the recent development of a star polycation-based RNA interference system that allows to penetrate the physical obstacles of eggshell and larval cuticle of *Spodoptera frugiperda* (Chao et al. 2023). This new technology will, in general, facilitate the effective delivery of dsRNAs targeting genes expressed during embryonic development, such as *Sl102*, which are good candidates for early pest suppression. The increasing available evidence that insect and mite eggs (Wang, 2011; Pampolini et al., 2020; Yang et al. 2024) can be permeated by dsRNA molecules and that the permeation rate can be remarkably enhanced provides new opportunities to develop early suppression strategies. Our results (phenotypic and molecular impact observed after *Sl102* gene silencing, quantitation of ds*GFP* entering eggs by qPCR analysis, and analysis of fluorescence intensity before and after washings of eggs soaked in labeled-ds*Sl102* solution; see Figs. 2, 3, 4, 5, and 6) reasonably support the hypothesis that nucleic acids, such as dsRNA molecules, can passively penetrate the chorion of insect eggs, once soaking concentrations and times are established. Even though eggs are multilayered and complex, to provide protection to the developing embryo, the multiple openings for respiration (aeropyles) and fertilization (micropyles) are potential entry sites for exogenous compounds (Campbell et al., 2016). In-depth studies on the mechanism of entrance involved will offer new insights on how to define new strategies for enhancing the uptake rate.

In conclusion, our results further expand the understanding of the *Sl102* gene function and provide experimental evidence supporting its important role during embryonic development of *S. littoralis*. The resulting developmental disruption induced by its silencing proves to be highly lethal and offers a new opportunity for developing more efficient pest control strategies, based on RNAi technology targeting precocious developmental stages for an early pest suppression.

Author contributions

Gennaro Volpe, investigation, formal analysis, validation, writing – original draft. Ilaria Di Lelio, conceptualization, investigation, formal analysis, validation, writing – original draft, writing – review & editing. Andrea Becchimanzi, validation, writing – review & editing. Daniele Bruno, investigation, writing – original draft, writing – review & editing. Eleonora Barra, investigation, formal analysis, validation. Elia Russo, investigation, validation. Giovanni Jesu, investigation, validation. Sabrina Di Giorgi, investigation, validation. Matteo Perrone, investigation, validation. Marco Gebiola, investigation, validation. Giulia Magoga, investigation, validation. Matteo Montagna, investigation, validation. Gianluca Tettamanti, conceptualization, writing – original draft writing – review & editing. Silvia Gigliotti, investigation, writing – review & editing. Francesco Pennacchio, conceptualization, writing – original draft, writing – review & editing funding acquisition, project administration, resources, supervision, validation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-025-01932-z.

Acknowledgements We thank Angharad Gatehouse (Newcastle University, UK) for her comments on the manuscript. This work was supported by: i. the European Union Horizon 2020 Research and Innovation Program (EcoStack; grant agreement no. 773554); ii. the Italian Ministry of University and Research PRIN 2020, no. 2020T58TA3 (BiPP). iii. the European Union Next-Generation EU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022).

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